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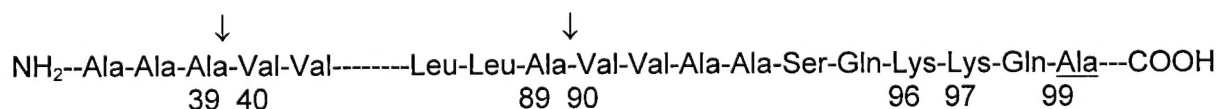
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## INTRODUCTION

The broad aim of this proposal was to test the hypothesis that the coordinate action of proteases and protease inhibitors are responsible for growth factor activation and release in the human breast cancer cell, and for the progression of breast cancer *in vivo*. It is well established that proteolytic enzymes play a significant role in the expression of the malignant phenotype including the loss of growth regulation, invasiveness and formation of metastases (Chintala et al 1999, Kleiner & Stetler-Stevenson 1999, Murphy & Gavrilovic 1999, Koblinski et al 2000). Tumor cell derived proteases have multiple activities and have been shown to degrade basement membrane components, stimulate angiogenesis, and promote tumor cell proliferation and migration. Proteases may also play a role in tumorigenesis without being directly involved with invasion and metastasis by enhancing the availability of soluble polypeptide growth factors. Several of these growth factors including the epidermal growth factor (EGF) and its analogs, heparin-binding epidermal growth factor (HB-EGF) and transforming growth factor- $\alpha$  (TGF $\alpha$ ), the insulin-like growth factors (IGF-I and IGF-II), mast cell growth factor and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and its receptor (Massagué & Pandiella 1993, Baselga et al 1996, Hooper et al 1997, Yavelow et al 1997, Kiessling & Gordon 1998), have been shown to require pericellular proteolysis for activation or release. To achieve homeostasis, levels of the growth-modulating proteases must also be regulated. This may occur through the action of locally synthesized protease inhibitors, although the mechanisms governing this process are unclear (Yavelow et al, 1997).

TGF $\alpha$ , a peptide structurally and functionally related to EGF, interacts with the EGF receptor (EGFR) and elicits a mitogenic response in a variety of cells (Lee et al 1995). Expression of the TGF $\alpha$ /EGFR pair have been shown to be associated with proliferation, migration and angiogenesis in invasive breast cancer (De Jong et al 1998) and in gliomas (Tang et al 1999). TGF $\alpha$  is synthesized as part of a 20-22 kDa glycosylated, type I membrane protein precursor (proTGF $\alpha$ ) which can be processed intracellularly and extracellularly by glycosylation and proteolysis to yield a family of polypeptides of from 6 to 17 kDa in size (Massagué & Pandiella 1993, Baselga et al, 1996). Proteolytic processing (**Scheme 1**) occurs in two steps, the first results in cleavage between Ala<sup>39</sup> and Val<sup>40</sup>. The second, occurring closer to the cell membrane, results in release of the 6 kDa mature TGF $\alpha$  peptide. While it is generally accepted that the second cleavage occurs between Ala<sup>89</sup> and Val<sup>90</sup>, this does not rule out cleavage at another site (e.g between Lys<sup>96</sup> and Lys<sup>97</sup>). Complete processing does not occur to the same extent in all tissues and it is certainly possible that shedding of TGF $\alpha$  from the cell surface may be accomplished by different proteases in different tissues (Hooper et al 1997, Arribas et al 1996). In agreement with this, TGF $\alpha$  release has been shown to be blocked by inhibitors of both metallo- and serine proteases (Pandiella et al 1992, Arribas et al 1996).



**Scheme 1. Cleavage sites in ProTGF $\alpha$  yielding mature 50 aa TGF $\alpha$  peptide. Arrows indicate the normal cleavage sites. Ala<sup>99</sup> begins the hydrophobic membrane-spanning region.**

Recently, it has been demonstrated that tumor necrosis factor- $\alpha$  converting enzyme (TACE, ADAM 17, MDC), the zinc metalloprotease responsible, at least in part, for release of TNF $\alpha$ , also plays a role in the release of TGF $\alpha$  and several other membrane proteins (Black et al 1997, Peschon et al 1998). The ADAMs (A Disintegrin And Metalloproteinase) are a family of multi-domain, adhesive, type-I transmembrane proteins with protease activity containing, disintegrin, metalloproteinase, cysteine-rich and EGF-like domains (Primakoff et al 2000). While both serine and metalloproteases have been shown to be involved in a wide range of biological processes including fertilization, neurogenesis, myogenesis and the inflammatory response, their exact roles for in these processes are unclear.

A relationship between tumorigenicity and endogenous synthesis of the protease inhibitor  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT) has been demonstrated in MCF-7 human breast cancer cells (Tamir et al 1990, Finlay et al 1993, Yavelow et al 1997). Release of TGF $\alpha$  from the cell surface was blocked by endogenously synthesized  $\alpha_1$ -AT. Consistent with this, a serine protease capable of forming a stable complex with  $\alpha_1$ -AT was located on the cell surface. To identify the  $\alpha_1$ -AT-sensitive TGF $\alpha$  cleavage enzyme from MCF-7 cells, degenerate oligonucleotides based on the conserved sequences about the his<sup>57</sup> and ser<sup>195</sup> residues in mammalian serine proteases were used to clone a series of cDNAs. One such cDNA encoded a novel, type II membrane serine protease with an 855 amino acid open reading frame. While this work was in progress, the cloning of a cDNA encoding a similar protease from mouse thymocytes, epithin, as well as an apparently identical protein from human prostate cancer cells, MT-SP1, was reported (Kin et al 1999, Takeuchi et al 1999). Matriptase, a recently described cDNA from T47-D breast cancer cells may be a partial epithin clone (Lin et al 1999). In this communication, we show that an antibody to the human epithin active site domain inhibits both release of TGF $\alpha$  from MCF-7 cells and invasion of BT-20 breast cancer cells through Matrigel. In addition, doubly-transfected MCF-7 cells hyper-expressing both proTGF $\alpha$  and epithin/MT-SP1 released significantly greater amounts of TGF $\alpha$  than non-transfected cells or MCF-7 cells transfected with proTGF $\alpha$  or epithin/MT-SP1 alone, confirming that epithin/MT-SP1 is involved either directly or indirectly in the processing of proTGF $\alpha$ .

Results from this laboratory have clearly demonstrated a relationship between tumorigenicity of MCF-7 human breast cancer cells (as measured by growth in soft agar), endogenous synthesis of the protease inhibitor  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT) and release of TGF $\alpha$  (Tamir et al 1990, Finlay et al 1993a,b). Growth in soft agar was blocked by  $\alpha_1$ -AT whether added to the tissue culture media or synthesized by the tumor cell itself. A useful tool in these studies was a new MCF-7 cell subline, producing 10-fold higher levels of  $\alpha_1$ -AT than its parental cell line, constructed by stable transfection with an  $\alpha_1$ -AT cDNA (Yavelow et al 1997). Growth in soft agar and release of TGF $\alpha$  was decreased in cells transfected with the  $\alpha_1$ -AT cDNA when compared to cells transfected with vector alone. Consistent with the above we had identified a serine protease with elastase-like activity, capable of forming a stable complex with  $\alpha_1$ -AT, on the MCF-7 cell surface.

Our specific aims as stated in our grant application were to:

1. To identify and clone the growth-modulating pericellular proteases from MCF-7 cells, particularly, the elastase-like enzyme(s) that are able to effect the release of TGF $\alpha$  from the tumor cell surface.
2. To show that the ability of MCF-7 sublines to form colonies in soft agar and tumors in nude mice is a function of their expression of  $\alpha_1$ -AT and specific pericellular proteases.
3. To extend our observations relating to TGF $\alpha$  release/activation made in MCF-7 human breast cancer cells in culture to other breast cancer cell lines, and to normal and malignant human breast tissue.
4. To test the hypothesis that the pericellular protease:protease inhibitor ratio responsible for TGF $\alpha$  release can be regulated by ST-3 and other potential effectors such as anti-estrogens, phorbol esters and SEC receptor agonists. This information may provide insight into mechanisms by which protease and protease inhibitor levels may be independently controlled.

To accomplish the above specific aims, we proposed to carry out the sequence of studies described in our Statement of Work. While the project has not proceeded in exactly the order as originally anticipated, we did accomplish most of what we set out to do as detailed in this report.

## YEAR

## STATUS OF COMPLETED STUDY

- 1-2 Isolate, characterize and clone proteases from MCF-7 cells. This phase of the work has been completed. A revised manuscript describing the cloning and properties of the TGF $\alpha$  cleavage protease (which we now call human epithin) has been submitted for publication.
- 1-3 Compare production of protease and protease inhibitors by MCF-7 cell sublines with their ability to form colonies in soft agar and cause tumor formation in nude mice. Studies of colony formation in soft agar and tumor foundation in nude mice have been completed.
- 1-3 Extend our observations relating to TGF $\alpha$  release/activation made in MCF-7 human breast cancer cells in culture to other breast cancer cell lines, and to normal and malignant human breast tissue. We have demonstrated production of PCR-7 protease in normal and malignant breast epithelial tissue and anticipate looking at TGF $\alpha$  shedding in other breast cancer cell lines and in breast epithelial tissue over the next several months. Breast cancer cell studies using the non-transformed cell line MCF-10A, the ER-negative cell lines BT-20 and T47D and a second ER-positive cell line, ZR-65-1 have been completed.
- 2-4 Examine production and localization of proteases, protease inhibitors, growth factor receptors and sites of growth factor activation in normal and malignant breast tissue. In collaboration with Dr. Helen Feiner (Department of Anatomic Pathology) we have compared the histochemical localization of epithin, EGF receptor and ProTGF $\alpha$  in normal and malignant breast tissue.
- 1-4 Test the hypothesis that the pericellular protease:protease inhibitor ratio responsible for TGF $\alpha$  release can be regulated by ST-3 and other potential effectors Identify potential modulators of protease inhibitor: protease ratio and growth factor activation/release in breast cancer cells and in ductal epithelium from human breast.

## BODY

### **1. Cloning of human epithin/MT-SP1: a potential TGF $\alpha$ releasing serine protease.**

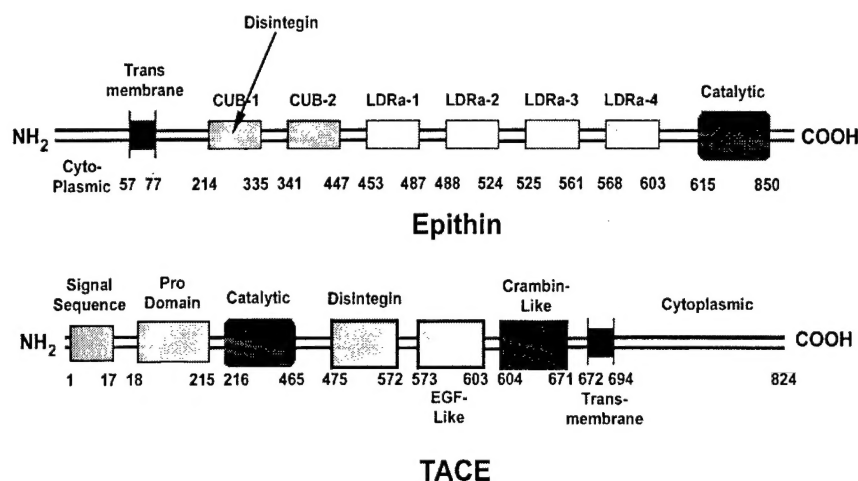
Total cellular RNA was prepared from MCF-7 (ML) cells by extraction in guanidinium isothiocyanate and centrifugation through cesium chloride (Chirgwin et al 1979). mRNA isolated using the PolyATtract mRNA isolation system IV (Promega) was used to synthesize double stranded cDNA (BRL cDNA Synthesis System). Degenerate oligonucleotide (19mer) primers designed from the conserved amino acids around the active site residues his<sup>57</sup> and ser<sup>195</sup> and from the conserved sequence around asp<sup>102</sup> (Sakanari et al, 1989; Elvin et al, 1993) were obtained from Gibco BRL. cDNA was amplified using the his<sup>57</sup> and ser<sup>195</sup> primers essentially as described by Elvin et al (1993). The PCR products were ligated directly into the TA cloning vector pCR<sup>TM</sup>II (Invitrogen) which was used to transform E. coli strain INV $\alpha$ F' (Invitrogen). Recombinant clones were selected by blue/white color selection on X-gal-containing agar plates. Plasmid DNA, isolated from 22 white colonies by alkaline lysis, was digested with Eco RI and electrophoresed on an agarose gel. After transfer to GeneScreen nylon membrane (NEN), the cDNAs were hybridized with the degenerate Asp<sup>102</sup> probes end-labeled with  $\gamma$ -<sup>32</sup>P ATP. The 460 bp sequence of one strongly hybridizing clone, designated PCR-7, showed a high degree of homology to known serine proteases (64% identity in a 220 bp region at the 3' terminus to human trypsinogen-B and a 60% identity in a 120 bp region around the 5' terminus to human pancreatic protease). Northern blot analysis, using a PCR-7 cDNA probe, showed the expression of an approximately 4.2 kb mRNA species in total RNA from MCF-7 cells. Similar mRNA species were expressed in a several different human breast cancer cell lines and in normal human breast tissue, in trophoblast from term human placenta and in proliferative phase uterine glandular epithelium. (**Fig. 1, 1999 Report**).



An MCF-7 cell cDNA library was constructed directionally (Not I-Sal I cut) in the vector pSport 1 using the SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning (Life Technologies). Recombinant colonies were screened by standard hybridization techniques using a 300bp fragment of the 460 bp PCR-7 cDNA as a probe. Two cDNAs of approximately 3.8 and 3.2 kb containing the PCR-7 sequence were isolated. Except for an additional 600 nucleotides in the 5' terminus of the larger clone and some minor differences in the 3' sequence, sequences of the two cDNAs appear to be identical where they overlap. The larger cDNA has a 2565 b open reading frame coding for an 855 amino acid protein of approximately 95 kDa and a 542 b 5' sequence following the stop codon at position 2721. (**Fig. 2, 1999 Report**). Using BLAST, an 80.3% identity in an 843 amino acid overlap was found between the deduced amino acid sequence and the deduced sequence of epithin a putative protease cloned from a mouse thymocyte cDNA library (Kim et al 1999). Recently a highly similar protease, MT-SP1 has been cloned from PC-3 human prostatic cancer cells (Takeuchi et al 1999). An almost perfect identity at the cDNA level was also found between the MCF-7 cell protease and matriptase, a 683 amino acid serine protease recently cloned from T47-D breast cancer cells (Lin et al 1999) suggesting that matriptase is a partial epithin clone.

The deduced amino acid sequence from the open reading frame contains the His<sup>57</sup>, Asp<sup>102</sup> and Ser<sup>195</sup> motifs characteristic of serine proteases (Val<sup>652</sup>-Cys<sup>657</sup>, Asp<sup>711</sup>-Leu<sup>714</sup> and Asp<sup>799</sup>-Ser<sup>810</sup> respectively) and is compatible with the S1 family of the SA clan of serine-type peptidases (Rawlings and Barrett, 1994). A potential serine protease activation sequence (RVVGG) is present at residues R<sup>614</sup>-G<sup>618</sup> suggesting that human epithin is synthesized as a single chain zymogen. While there is no apparent signal peptide sequence, Kyle-Doolittle hydropathy plots using the GCG Peptide Structure program (**Fig. 3, 1999 Report**) indicated the presence of a highly hydrophobic region between residues V<sup>57</sup> and W<sup>77</sup> consistent with a transmembrane spanning region. Similar results were obtained using the TMpred (Hofman & Stoffel, 1993) and TopPred 2 programs (Claros & von Heijne, 1994). A ProfileScan of the ORF against the Prosite library showed two potential CUB domains (residues 214-334 and 340-447) and four potential LDL-receptor class A domains (residues 452-487, 487-524, 524-560 and 566-603). Epithin has 40 putative external cysteine residues. Each LDR repeat contains six cysteine residues presumably in 3 internal disulfide linkages (Brown et al 1997). Each of the two CUB domains contains four cysteine residues in disulfide linkage that, also most likely are in internal disulfide linkages (Bork & Beckman 1993). The catalytic domain has 8 cysteine residues in four disulfide linkages analogous to chymotrypsin (i.e. Cys<sup>604</sup>-Cys<sup>732</sup>, Cys<sup>641</sup>-Cys<sup>657</sup>, Cys<sup>776</sup>-Cys<sup>790</sup>, Cys<sup>801</sup>-Cys<sup>830</sup>). Cys<sup>604</sup>-Cys<sup>732</sup> serves to link the catalytic and regulatory domains. The domain structure and properties of epithin appear to be analogous to those of TACE, a matrix metalloproteases of the ADAMs family. Both are trans membrane proteins (although of different types) containing extensive cysteine-rich regions (crambin-like domain in TACE; CUB and LDRa domains in epithin) and have similar functions (shedase and potential disintegrin activities). It is possible that both TACE and epithin can lose their protease activities while maintaining their adhesive properties. Schematic representations of the human epithin and TACE domain structures are compared in **Fig. 1**.

Using BLAST, an 80.3% identity in 843 amino acid overlap was found between the deduced amino acid sequence and the deduced sequence of epithin a putative protease cloned from a mouse thymocyte cDNA library (Kim et al 1999). An almost perfect identity at the cDNA level was found between the MCF-7 cell protease and Matriptase, a 683 amino acid serine protease recently cloned from T47-D breast cancer cells (Lin et al 1999). A 45-55% identity at the amino acid level was found between the human epithin and the human serine proteases (or their zymogens) enterokinase precursor (Kitamoto et al 1995), hepsin (Leytus et al 1988), prekalikrein (Chung et al 1986), TMPRSS2 protease (Paolino-Giacobino, et al 1997), prostasin (Yu et al 1995) and drosophila protease stubble (Appel et al 1993). Significantly, enterokinase precursor, hepsin, prekalikrein, and prostasin are all cell membrane-bound proteases. Epithin most likely shows specificity for cleavage of peptide bonds after Lys or Arg residues as it, like trypsin, hepsin and enterokinase contains an Asp (Asp<sup>266</sup>) at the base of the specificity pocket (S1 subsite). Elastase and chymotrypsin-like enzymes have cysteine and serine residues, respectively, at this site.



**Figure 1.** Comparison of Human Epithin and TACE Domain Structures

## **2. Construction of MCF-7 cell sublines demonstrating stable expression of human epithin and proTGF $\alpha$**

The plasmid pSport 1 containing human epithin cDNA in the Not I – Sal I site was digested with Sal I. After filling in the ends with DNA polymerase 1 (Klenow) and ligating Hind III linkers the plasmid was digested with Hind III and Not I. This fragment was purified and ligated to the Hind III – Not I site of the eukaryotic expression vector pRC/CMV (Invitrogen) which contains the gene encoding for neomycin resistance (neo<sup>r</sup>). This strategy ensured that the insert is in the correct orientation for expression. MCF-7 (ML) cells were transfected with this construct by the calcium phosphate technique. After transfection the cells were plated at low density and cultured for approximately 14 days in medium containing 550  $\mu$ g/ml Geneticin (G418). The media was changed every 4-5 days to remove dead cells. G418 resistant colonies were trypsinized using cloning rings and transferred to 15 mm wells. 6 of these colonies were selected for expansion and Northern blot analysis. 3 of the 6 clones clearly exhibited overexpression of epithin mRNA.

The plasmid pHGF1 – 10 – 925, containing the human proTGF $\alpha$  cDNA, was obtained from American Type Culture Collection (Rockville, Maryland). The 925 bp Eco RI insert includes 49 bp of 5' untranslated sequence, 480 bp encoding TGF $\alpha$  precursor and 391 bp 3' untranslated sequence. After filling in the ends with DNA polymerase I (Klenow) and ligating Hind III linkers the insert was ligated into the Hind III site of the eukaryotic expression vector pcDNA3.1/Hygro(+). This vector confers resistance to hygromycin and can be selected for independently in cell lines that have been transfected with the neomycin resistance gene. The orientation of the insert was determined by restriction digest. MCF-7 (ML) and MCF-7 (ML) cells previously transfected with epithin cDNA were transfected by the calcium phosphate coprecipitation method and selected with hygromycin (80  $\mu$ g/ml for ML cells, 20  $\mu$ g/ml for epithin transfected ML cells). One colony of each cell line exhibiting overexpression of TGF $\alpha$  by Northern blot analysis was selected for further study. A similar methodology was used to construct an MCF-7 subline expressing high levels of expressing high levels of both epithin and TGF $\alpha$ .

## **3. Activation and release of human epithin from MCF-7 cells**

Western blotting showed that both the parental MCF-7 subline and a subline stably transfected with a full-length from epithin cDNA release the same amount of an approximately 84 kDa fragment into the media (**Fig. 4, 1999 report**). The nature of the fragment is unclear although it must contain the catalytic domain as this region was used to prepare the antibody. Whether the fragment is generated by proteolytic cleavage or results from altered splicing is presently under investigation, although the later possibility appears to be remote as only a single 4.2 kb transcript is seen on Northern blots. There is a prominent 33 kDa band in reduced samples in the spent media from transfected cells which is barely visible in spent media from the parental cells suggesting that it may be a consequence of autoprocessing. Undoubtedly, this is the catalytic domain as membrane fractions from MCF-7 cells

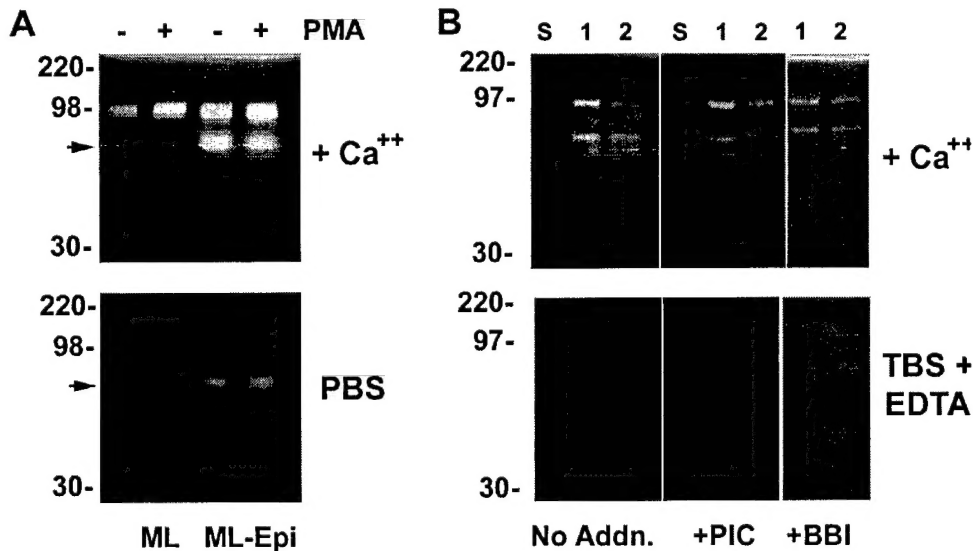


labeled with the serine protease inhibitor [ $^3\text{H}$ ]-diisopropyl fluorophosphate, showed a 33 kDa [ $^3\text{H}$ ]-labeled band, which also was recognized by the epithin antibody after western blotting (**Fig. 5, 1999 Report**). The fact that treatment with phorbol 12-myristate 13-acetate (PMA) was without effect suggests that epithin activation and release of TGF $\alpha$  may not be directly connected. The observation that addition of the serine protease inhibitor phenylmethylsulfonyl fluoride (DIFP) to the spent media after collection also had no effect suggests that activation occurs rapidly.

Western blotting of spent media from MCF-7 cells recognized an approximately 45 kDa polypeptide without, and 33 kDa with reduction. The 45 kDa fragment most likely contains the activated catalytic chain and an approximately 12 kDa fragment from the regulatory chain. The nature of the prominent 45 kDa band seen in all of the membrane fractions, and whether it is identical to the slightly larger poly peptide seen in the spent media is presently under investigation. Significantly, in spent media from cells incubated with the metalloprotease inhibitor BB-3103, only a 84 kDa band is evident suggesting a metalloprotease may be needed for initial epithin activation.

#### 4. Epithin shows gelatinase activity

Gelatin zymography was used to confirm that human epithin, like matriptase has activity against matrix proteins (**Fig. 2**). In the presence of  $\text{Ca}^{++}$ , required for the action of metalloproteases, two bands are apparent (**Fig. 2 left panel**) in both the parental and epithin-transfected cell lines.



**Figure 2. Epithin gelatinase activity in spent media from MCF-7 cells.** A. Confluent MCF-7 ML (ML) cells and MCF-7 ML cells transfected with a full-length epithin cDNA (ML-Epi) were incubated for 21 h in serum-free DMEM media containing  $10^{-8}$  M estradiol. PMA (50 ng/ml) was added to half the flasks and the incubation was continued for an additional 4 h. Conditioned media was centrifuged and the supernatant brought to 65% saturation with ammonium sulfate. After centrifugation,

precipitates were solubilized in 10 mM phosphate buffer (pH 6.8) and dialyzed overnight. Aliquots were electrophoresed on 10% SDS polyacrylamide gelatin zymogram gels without reduction. Gels were washed with several changes of 2.5% Triton-X-100 and incubated at 37° overnight in 50mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.02% Brij-35 and either 5mM  $\text{CaCl}_2$  or 10 mM EDTA. Gels were then stained with Coomassie Blue R-250.

B. MCF-7 cells transfected with a full-length human epithin cDNA (ML-Epi) were incubated overnight in serum-free DMEM containing  $10^{-8}$  M estradiol. Conditioned media were concentrated in Centricon YM-3 concentrators (Millipore) with (lane 1) or without (lane 2) ammonium sulfate precipitation. Aliquots were electrophoresed on 10% gelatin zymogram gels. Gels were washed with several changes of 2.5% Triton-X-100 and incubated for 18 hours at 37° C in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.02% Brij-35 with either 5mM  $\text{CaCl}_2$  or 10 mM EDTA, containing either Boehringer Mannheim, mini EDTA-free protease inhibitor cocktail (PIC) or 100  $\mu\text{g/ml}$  Bowman-Birk soy bean protease inhibitor (BBI).

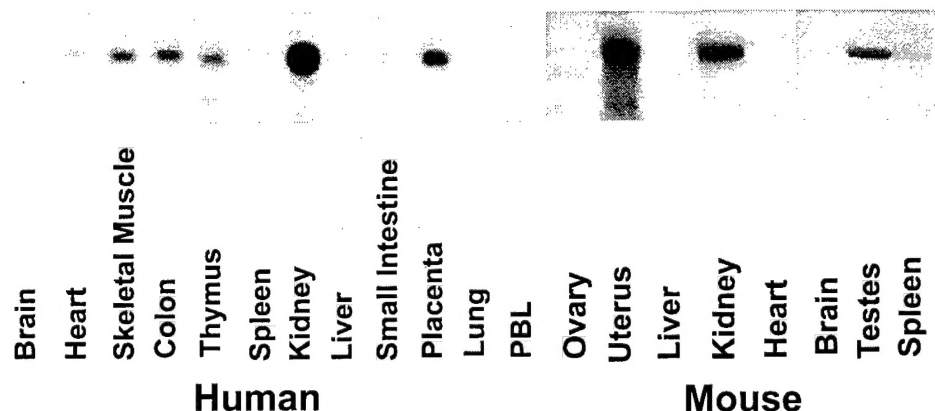
The higher, 92 kDa band is present at the level in both cell lines. The lower, 84 kDa band, is considerably more pronounced in the transfected cells. When the zymograms were incubated in the presence of EDTA, an inhibitor of matrix metalloproteases, the upper band, most likely the MMP9 gelatinase, disappeared. This would suggest that the lower band is epithin or some other non-metalloprotease. That this is the case is shown in the **right panel** where the 84 kDa band was made to disappear by incubation with DIFP whether in the presence or absence of EDTA. Consistent with the

western blotting experiments, PMA had no effect. The observation that the 84 kDa activity is inhibited by DIPF but not by EDTA and that it is elevated in transfected cells strongly suggests that the 84 kDa activity is indeed epithin and that epithin may be directly involved in invasion.

### 5. Epithin gene is conserved in mammalian species.

Southern analysis of genomic DNAs after cleavage with either EcoR1 or Pst-1 and probed with a PCR-7 cDNA, indicated the presence of homologous sequences in human, mouse, baboon, rat and rabbit but not in chicken or drosophila genomic DNA (Fig. 6, 1998 Report). Using this same probe, expression of mRNA species similar in size to that found in MCF-7 cells, human breast and uterine epithelium and in trophoblast was demonstrated in mouse testes, kidney, ovary and uterus (Fig. 3).

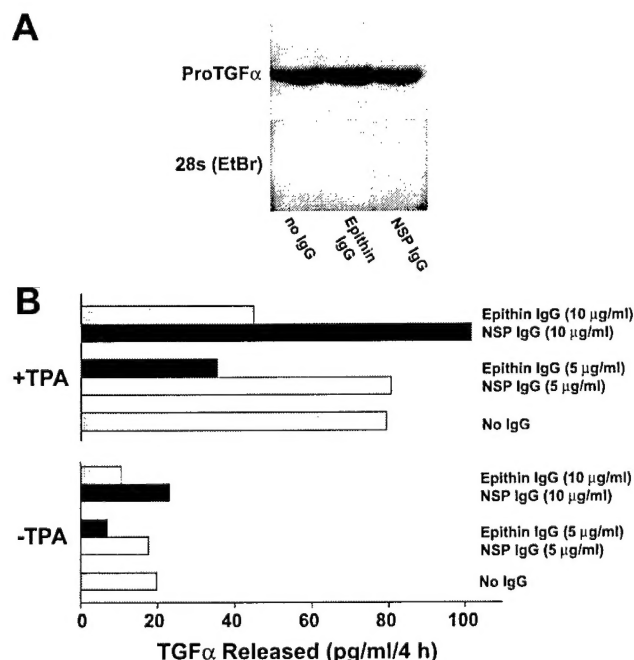
RNA from mouse breast or placenta was not tested.



**Figure 3. Northern blot analysis of epithin mRNA in various mouse and human tissues.** The blot was screened with a  $^{32}$ P-labeled 300 bp fragment (nt x-y) of the full-length Epithin cDNA generated by PCR.

### 6. Rabbit antibodies to human epithin inhibit $TGF\alpha$ release from MCF-7 breast cancer cells.

An antibody to the active site region of human epithin was raised in rabbits (1998 Report). Antibody does not seem to recognize antigen on live MCF-7 cells in culture (but does recognize antigen on paraffin-embedded fixed cells). The antibody inhibits TPA-induced  $TGF\alpha$  release from MCF-7 cells in a dose-dependent manner as compared to pre-immune sera (Fig. 4). These results suggest that one function of epithin is to catalyze release of  $TGF\alpha$ .

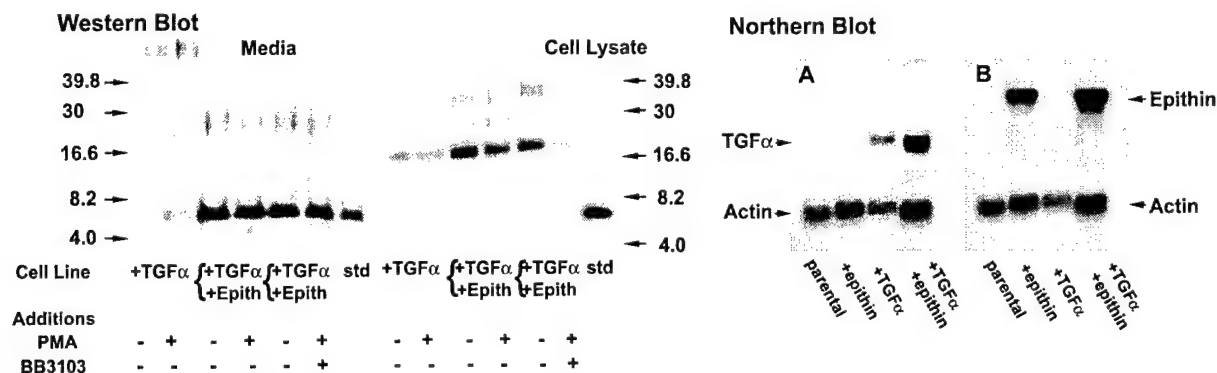


**Figure 4. Antibody to human epithin inhibits release of  $TGF\alpha$  from MCF-7 cells.**

MCF-7 (ML) cells were incubated in serum-free media containing  $10^{-8}$ M E2 for 18 h. Cells were then incubated in fresh serum-free media containing antibody (IgG fraction) to the epithin active site region (Epithin IgG) or non-immune IgG (NSP IgG) at the concentrations indicated. Control cells were incubated without added IgG (No IgG). After 30 min, PMA (50 ng/ml) was added and the cells were incubated for an additional 4 hours. **A:** ProTGF $\alpha$  mRNA in total RNA isolated from the samples, was determined by Northern blot analysis. **B:** TGF $\alpha$  levels in conditioned were determined using an ELISA assay (Oncogene Science) according to the manufacturer's instructions. Samples were concentrated by reverse phase chromatography, taken to dryness and then resuspended in PBS prior to assay.

## 7. TGF $\alpha$ release is highest in transfected MCF-7 cell sublines expressing high levels of both proTGF $\alpha$ and epithin.

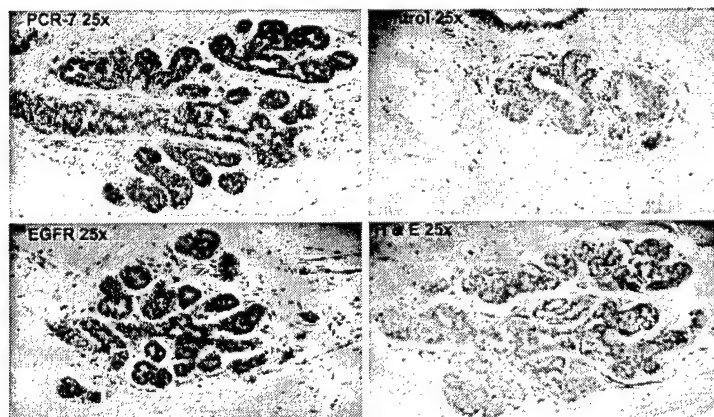
To demonstrate directly that epithin could catalyze release of TGF $\alpha$  from the MCF-7 cell surface, a series of MCF-7 cell sublines expressing elevated levels of proTGF $\alpha$ , epithin and epithin plus proTGF $\alpha$  were generated by the methods described in section 2. Northern blot analysis indicates that the doubly-transfected epithin/TGF $\alpha$  subline expresses the same level of epithin as the singly-transfected epithin subline or the singly transfected TGF $\alpha$  subline. Western blot analysis indicates that the release of 6kDa TGF $\alpha$  by the doubly-transfected subline is 5-fold greater then by the singly-transfected TGF $\alpha$  subline. These results confirm that epithin can release TGF $\alpha$  from the cell surface.



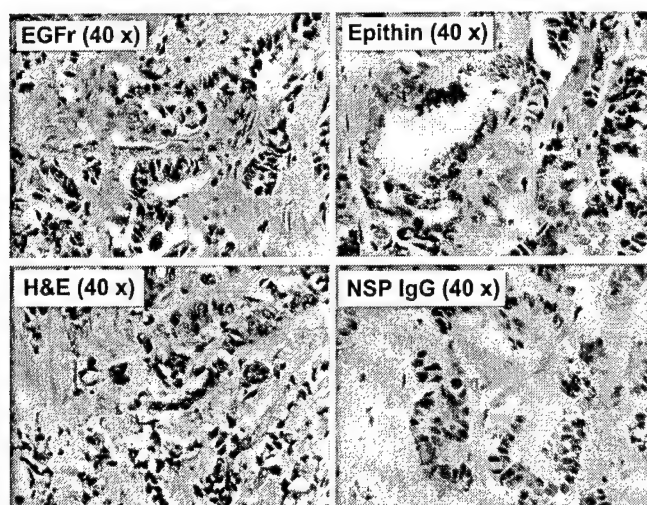
**Figure 5. TGF $\alpha$  release in transfected MCF-7 cell sublines.** **Western blot:** Transfected MCF-7 (ML) parent cells and cells transfected with human epithin cDNA were transfected with human TGF- $\alpha$  cDNA. Confluent T25 flasks were washed 2X with serum-free DMEM and incubated for 21 hours with serum-free DMEM containing  $10^{-8}$  M estradiol. BB 3103 ( $1\mu\text{M}$ ) and PMA ( $100\text{ng/ml}$ ) were added as indicated and the cells were incubated an additional 4 hours. Media were collected, centrifuged to remove loose cells, and concentrated in Centricon YM-3 concentrators (Millipore). Cells were washed once with PBS and lysed in PBS/TDS containing  $0.1\text{mM}$  PMSF. Aliquots were electrophoresed on 16.5% Tris-Tricine gels under denaturing conditions, transferred to PVDF membranes and subjected to Western blot analysis using a monoclonal antibody to TGF $\alpha$ . After incubation with horseradish peroxidase-conjugated second antibody the signals were visualized by chemiluminescence. Std: standard TGF $\alpha$ . **Northern Blot:** Total RNA isolated from ML cells (parental) and ML cells transfected with epithin and/or TGF $\alpha$  were subjected to Northern blot analysis using  $^{32}\text{P}$ -labeled cDNA probes for TGF $\alpha$  and epithin as indicated. Blots were then stripped and reprobed with  $\beta$ -actin.

## 7. Co-localization of human epithin and EGFr to the outer surfaces of normal breast ductal epithelial and breast cancer cells.

Immunohistochemistry of paraffin sections from normal human breast tissue showed that the epithin protein was restricted to the surface of ductal epithelial cells where it co-localized with the epidermal growth factor receptor (Fig. 6a,b). In addition, epithin expression appeared to be elevated in DCIS and in invasive breast carcinoma (Fig. 6b).



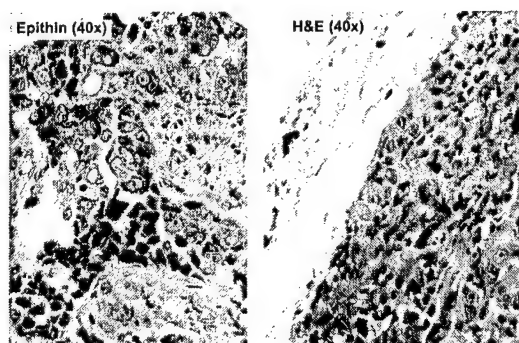
**Figure 6a. Immunohistochemical co-localization of human epithin and EGFr to outer surfaces of breast ductal epithelial cells.** Five  $\mu\text{m}$  paraffin-embedded tissue sections from a normal region of a benign breast tumor were screened with either a rabbit polyclonal epithin (PCR-7) antibody, the same amount of pre-immune IgG or a mouse monoclonal anti-human EGFr antibody. Treatment with antirabbit or antimouse peroxidase conjugate and color development with DAB were carried out using a Vectastain ABC kit. Samples were counterstained with hematoxylin and eosin for morphology.



**Figure 6b. Immunohistochemical co-localization of human epithin and EGFr in human breast cancer.** Protocol was the same as described in 1a above. The pathologist described this as highly invasive, with regions DCIS. Both EGFr and epithin show highly positive staining in the epithelium and stroma of the tumor-containing regions. However, in the stroma of non-tumor regions, staining for both appears to be negative.

#### **8. Expression of the membrane serine protease epithin and the serine protease inhibitor, $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT), by MCF-7 breast cancer cells may regulate tumorigenesis.**

We have shown that expression of high levels of the serine protease inhibitor  $\alpha_1$ -AT by MCF-7 cells, inhibits release of TGF $\alpha$  and blocks anchorage-independent growth in soft agar. Consistent with this, we have identified a membrane anchored serine protease on MCF-7 cells, epithin, which appears to play a role in TGF $\alpha$  release. The tumor-forming ability of the MCF-7 parental cell line and sublines generated by transfection with an expression vector containing full-length cDNAs for human epithin,  $\alpha_1$ -AT and  $\alpha_1$ -AT<sub>Pittsburgh</sub> (an  $\alpha_1$ -AT variant in which Ser359 at the inhibitory site was replaced by arginine) were compared. While  $\alpha_1$ -AT can inhibit both elastase-like and trypsin-like enzymes,  $\alpha_1$ -AT<sub>Pittsburgh</sub> can only inhibit trypsin-like enzymes. Four to five week old female nude mice (NCRNU-M, Taconic Farms) were implanted subcutaneously with a continuous release pellet containing either placebo or 17 $\beta$ -estradiol (1.7 mg, 60 day release, Innovative Research of America). MCF-7 (ML) cells and the sublines stably transfected with  $\alpha_1$ -AT,  $\alpha_1$ -AT<sub>Pittsburgh</sub> and epithin were maintained in DMEM containing 10% FBS. On the day of the experiment the cells were trypsinized, counted, washed once with serum free medium and suspended in sterile saline.  $2 \times 10^6$  viable cells in 0.2 ml were injected subcutaneously approximately 1 cm from the pellet on the left flank of each animal. For each cell line, 5 animals received estradiol and 5 received a placebo. After 34 days the animals were killed by CO<sub>2</sub> narcosis and the size of the visible tumors were measured. Paraffin sections from each of the tumors were stained with hematoxylin-eosin or else immunostained with non-specific IgG, and specific antibodies to human epithin, EGFr, TGF $\alpha$  and  $\alpha_1$ -AT (**Fig. 7**). Appearance of the stained tumors was consistent with that of malignant breast tumors of human origin. The parental cell line and the subline hyper-expressing  $\alpha_1$ -AT<sub>Pittsburgh</sub> both formed tumors in nude mice in the presence of E2 (**Table 1**). Epithin hyper-expressing MCF-7 cells formed tumors both in the presence and absence of E2. The subline hyper-expressing  $\alpha_1$ -AT failed to form tumors either in the presence or absence of E2.



**Figure 7. Immunohistochemical Staining of MCF-7 Cell Tumors in Nude Mice.**

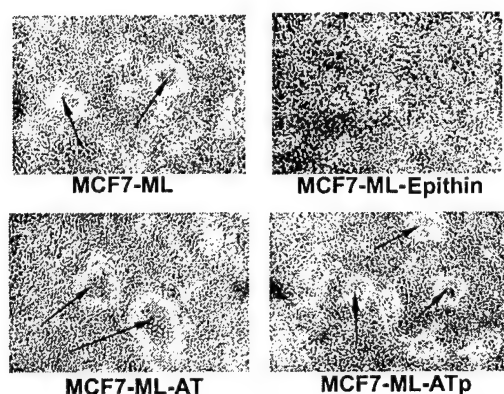
Five mm paraffin-embedded tissue sections from a tumor resulting from inoculations of MCF-7(ML) cells into a nude mouse along with a sustained E2-release pellet, were screened with either a rabbit polyclonal antibody to human epithin or the same amount of pre-immune IgG (data not shown). Treatment with anti-rabbit peroxidase conjugate and color development with DAB were carried out using a Vectastain ABC kit. A third section was stained with hematoxylin and eosin to show morphology.



**TABLE 1** Tumor formation in nude mice resulting from subcutaneous injection of MCF-cell lines in the presence or action of sustained release E2 pellets

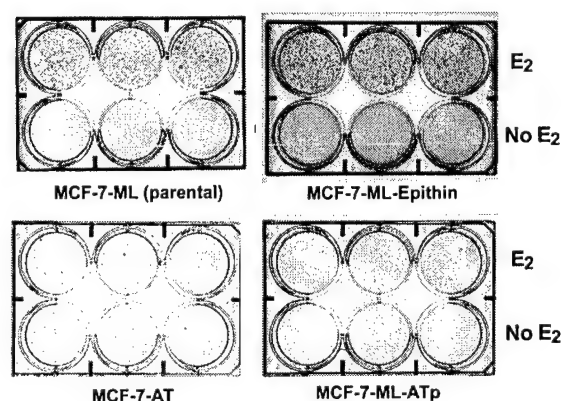
MCF-7 Cell Subline	Total Animals	Animals with Tumors
Control - E2	5	1
Control + E2	5	5
Epithin - E2	5	4
Epithin + E <sub>2</sub>	5	5
$\alpha_1$ -AT - E <sub>2</sub>	5	1
$\alpha_1$ -AT + E <sub>2</sub>	5	1
$\alpha_1$ -AT <sub>Pittsburgh</sub> - E <sub>2</sub>	5	0
$\alpha_1$ -AT <sub>Pittsburgh</sub> + E <sub>2</sub>	4	4

The parental MCF-7 (ML) and three derived sublines all grew well on plastic. However, post-confluent cultures of the MCF-7 (ML) and the protease inhibitor transfected sublines but not the epithin-transfected subline demonstrated "dome" formation (an indication of differentiation) (**Fig. 8**), an observation suggesting that elevated expression of epithin may cause de-differentiation of MCF-7 cells. Consistent with their ability to form tumors in nude mice, only the parental, epithin hyper-expressing sublines were able to form colonies in soft agar (**Fig. 9**). These results support our hypothesis that  $\alpha_1$ -AT and epithin may play a role in the regulation of tumorigenesis in breast epithelial cells.



**Figure 8 (left). Dome Formation by post-confluent cultures of MCF-7 cell sublines on plastic.** MCF-7 (ML) cells and the transfected sublines hyper-expressing  $\alpha_1$ -AT (AT),  $\alpha_1$ -AT<sub>Pittsburgh</sub> (ATp) and epithin were cultured in DMEM containing 10% fetal bovine serum. The media were changed every 3-4 days and the cells were allowed to grow past confluency (~5 days). Dome formation, indicated by arrows, (or lack of it in the case of the epithin-hyper-expressing subline) was consistent throughout passage of the individual cell lines.

**Figure 9. Anchorage-independent growth (colony formation) of MCF-7 cell sublines in soft agar.** Cells ( $10^4$ /30-mm tissue culture dish) from the MCF-7 cell sublines indicated above, were plated in RPMI-1640 media containing 10% charcoal-stripped FBS and 0.3% agar with and without  $10^{-8}$  M E2 over a bottom layer of 0.5% agar. Dishes were incubated at 37 C in atmosphere containing 5% CO<sub>2</sub>. After 21 days, cells were stained for 24 h with 3-(p-iodophenyl) 3-(p-nitrophenyl)- 5 phenyl-terazolium chloride and photographed.



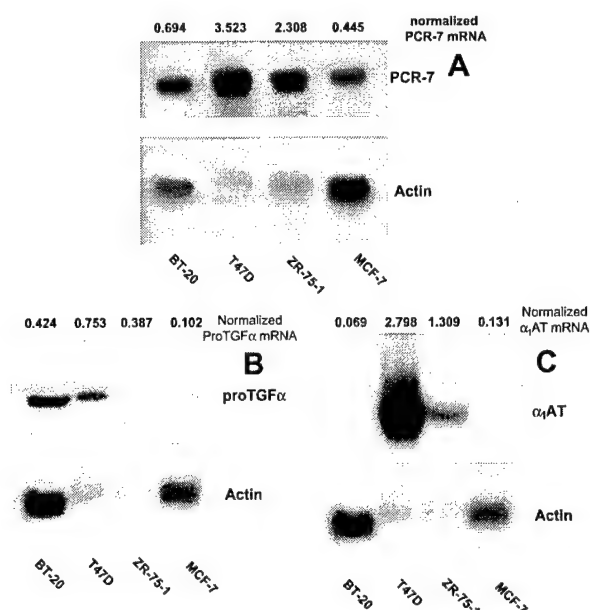
### 9. Invasion of breast cancer cells is regulated by epithin, TGF $\alpha$ and $\alpha_1$ -AT.

If our hypothesis that breast cancer cell invasion requires TGF $\alpha$  release (at least in part) and that release is a function of the relative levels of proteases and protease inhibitors is correct, then



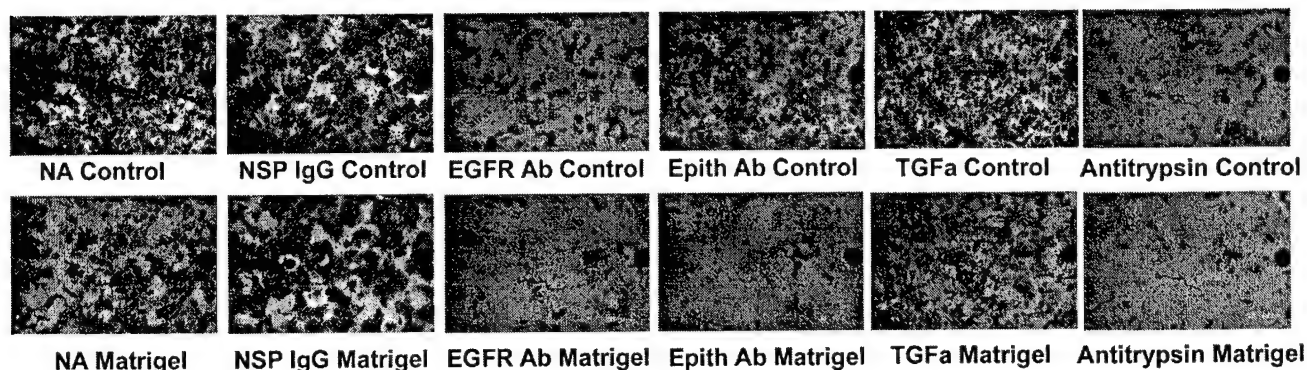
invasive breast cell lines should express proTGF $\alpha$ , a specific protease and a specific protease inhibitor. If epithin is the specific protease, and  $\alpha_1$ -AT, the specific inhibitor, TGF $\alpha$  release and invasion should be related the ratio of the expression of epithin:  $\alpha_1$ -AT. test this hypothesis we have first compared steady-state mRNA levels of epithin,  $\alpha_1$ -AT proTGF $\alpha$  in four different invasive human breast cancer cell lines, BT-20, T47D, ZR-75- and MCF-7 cells (Fig. 10).

**Figure 10 (right).** Steady state levels of epithin (PCR-7), proTGF $\alpha$  and  $\alpha_1$ -AT mRNA levels in human breast cancer cell lines.



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Consistent with their highly invasive nature, BT-20 cells demonstrate relatively high expression of epithin and proTGF $\alpha$  and relatively low expression of  $\alpha_1$ -AT (Fig. 10). For this reason, the ability of  $\alpha_1$ -AT, TGF $\alpha$  and antibodies to epithin and the EGFr were tested for their ability to modulate invasion of BT-20 cells through Matrigel (Fig. 11). The rabbit anti-EGFr anti-epithin antibodies both inhibited migration and invasion although, the effects of the EGFr antibody appeared to be more profound (Table II). Nonspecific rabbit IgG had no effect. Under these conditions, endogenous TGF $\alpha$  slightly stimulated invasion. Both migration and invasion were significantly inhibited by  $\alpha_1$ -AT providing additional evidence for our hypothesis that TGF $\alpha$ -mediated invasion of breast cancer cells can be regulated by epithin and  $\alpha_1$ -AT acting in concert.



**Figure 11. Invasion of BT-20 breast cancer through Matrigel.** BT-20 human breast cancer cells (0.5 ml) suspended at a density of  $10^5$  cells/ml in RPMI 1640 medium with 10% FBS were added to the upper well of modified Boyden chambers with 8.0  $\mu$  pore membranes (24 well, BIOCOAT, Becton Dickinson Labware). Migration (Control) was determined using uncoated membranes while invasion (Matrigel) was determined using membranes coated with growth factor reduced matrigel matrix (Becton Dickinson Labware) at a concentration of 85  $\mu$ g/cm<sup>2</sup> of membrane. Conditioned media from MCF-7 (ML) cells (0.75 ml) containing the same additions was added to the bottom well underneath the insert. After incubation for 48 hrs at 37 $^\circ$  C, the top surface of the membrane was cleared of cells with a cotton swab and cells that had penetrated to the bottom side of the membrane were fixed and stained using a Diff-Quik Stain kit (Dade Diagnostics). NA, no additions; NSP IgG, non-specific Rabbit IgG (2  $\mu$ g/ml); EGFR Ab, mouse monoclonal antibody to human EGF-R, 2  $\mu$ g/ml (Oncogene Research Products Ab-1); Epith Ab, rabbit polyclonal antibody to human epithin, 1.5  $\mu$ g/ml; TGF $\alpha$  (50 ng/ml),  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT, 100 $\mu$ g/ml).

**Table II Migration/Invasion of BT-20 Cells Through Soft Agar\***

Sample	Control (Migration)	Invasion
No Additions	100	100
Non-Specific IgG, 2 $\mu$ g/ml	105 $\pm$ 11	108 $\pm$ 13
EGFr Antibody, 2 $\mu$ g/ml	22.5 $\pm$	15.5 $\pm$ 6.3
Epithin Antibody, 1.5 $\mu$ g/ml	64.0 $\pm$	35.3 $\pm$ 5.7
TGF $\alpha$ , 50 ng/ml	108 $\pm$ 14	128 $\pm$ 9
$\alpha_1$ -AT, 100 $\mu$ g/ml	12.0 $\pm$ 8.0	14.5 $\pm$ 4.5

\* $\pm$  s.d. for 3 independent experiments

\*\* No additions considered to be 100%

## KEY RESEARCH ACCOMPLISHMENTS

- Degenerate oligonucleotides based on the conserved sequences about the his57 and ser195 residues in mammalian serine proteases were used to clone a membrane serine protease from MCF-7 breast cancer cells. The 3270 bp cDNA has a deduced 855 amino acid sequence consistent with that of a multi-domain, type II membrane protease and shows considerable homology to epithin, a recently described protein from mouse thymocytes. Mouse and human epithin have similar membrane spanning and trypsin-like serine protease domains. Each also has two putative CUB and four low-density lipoprotein receptor domains, which may be important for regulation and/or substrate binding. Matriptase, a recently described cDNA from T47-D breast cancer cells may be a partial epithin clone.
- Northern blotting showed highest expression of epithin in human epithelial cells particularly breast, kidney, trophoblast, and uterine glandular epithelium. Southern analysis indicated the presence of sequences homologous to human epithin in baboon, mouse, and rabbit but not in chicken or drosophila DNA.
- Western blotting of spent media from MCF-7 cells recognized an approximately 42 kDa polypeptide without, and 33 kDa with reduction. However, in media from cells incubated with the metalloprotease inhibitor BB3103, only a 84 kDa band was evident suggesting a metalloprotease may be needed for epithin activation. Membrane fractions from MCF-7 cells labeled with the serine protease inhibitor [ $^3$ H]-diisopropyl fluorophosphate, showed a 33 kDa [ $^3$ H]-labeled band, which also was recognized by the epithin antibody after western blotting. On gelatin zymography, two prominent bands were apparent in the presence of Ca $^{++}$ , the higher MW form disappeared in the presence of EDTA, an inhibitor of matrix metalloproteases, the lower in the presence of inhibitors of serine proteases. These results suggest that epithin may be directly involved in invasion.
- A polyclonal antibody to the epithin active site domain inhibited phorbol ester-induced TGF $\alpha$  release from MCF-7 cells by >50% suggesting that epithin is involved in proTGF $\alpha$  processing. This same antibody also inhibited invasion of BT-20 breast cancer cells through Matrigel.

- Immunohistochemistry of paraffin sections from normal human breast tissue showed that the epithin protein was restricted to the surface of ductal epithelial cells where it co-localized with the epidermal growth factor receptor. Epithin expression appeared to be elevated in DCIS and in invasive breast carcinoma.
- The MCF-7 parental cell line and sublines hyper-expressing epithin,  $\alpha_1$ -AT and  $\alpha_1$ -AT<sub>Pittsburg</sub> were constructed.
  - a. All grew well on plastic. However, the epithin hyper-expressing subline did not form "domes" suggesting a lesser degree of differentiation.
  - b. Only the parental, epithin and  $\alpha_1$ -AT<sub>Pittsburg</sub> hyper-expressing sublines were able to form colonies in soft agar.
  - c. The parental cell line and the subline hyper-expressing  $\alpha_1$ -AT<sub>Pittsburg</sub> both formed tumors in nude mice in the presence of E2. Epithin hyper-expressing MCF-7 cells formed tumors both in the presence and absence of E2. The subline hyper-expressing  $\alpha_1$ -AT failed to form tumors either in the presence or absence of E2.

These results support our hypothesis that epithin and  $\alpha_1$ -AT (an inhibitor of elastase-like enzymes) but not  $\alpha_1$ -AT<sub>Pittsburg</sub> (an inhibitor of thrombin-like enzymes) may play a role in the regulation of tumorigenesis in breast epithelial cells.

## REPORTABLE OUTCOMES

A 3270 bp cDNA containing an open reading frame coding for an 855 amino acid type II membrane serine protease was cloned from MCF-7 breast cancer cells. We have called the protease epithin because of its homology to a protease isolated from mouse thymocytes. While this work was in progress similar, if not identical, enzymes were cloned from mouse thymocytes, human prostate cancer cells and T47D human breast cells.

An antibody to the epithin active site was raised in rabbits. This antibody was shown to block both TGF $\alpha$  release from MCF-7 cells and invasion of BT-20 cells through Matrigel.

New MCF-7 cell sublines expressing high levels of TACE, epithin,  $\alpha_1$ -AT,  $\alpha_1$ -AT<sub>Pittsburg</sub>, TGF $\alpha$ , and epithin + TGF $\alpha$  were constructed.

Two manuscripts describing the above outcomes are in preparation.

## CONCLUSIONS

Elevated expression of the serine protease inhibitor  $\alpha_1$ -antitrypsin ( $\alpha_1$ -AT) by MCF-7 breast cancer cells is associated with decreased release of transforming growth factor- $\alpha$  (TGF $\alpha$ ) and reduced colony formation in soft agar. Consistent with this an 84 KDa, multi-domain, type II membrane serine protease, which we have called epithin, because of its homology to a recently described protein from mouse thymocytes, was cloned from MCF-7 cells. Northern blotting showed highest expression of epithin in mouse and human and epithelial cells particularly those from breast, kidney, trophoblast and uterine glands. Southern analysis indicated the presence of sequences homologous to human epithin in baboon, mouse, and rabbit but not in chicken or drosophila DNA. The domain structure and properties of epithin appear to be analogous to those of the ADAMs family of matrix metalloproteases. Immunohistochemistry of normal human breast tissue showed that the epithin protein was restricted to the surface of ductal epithelial cells where it co-localized with the EGFr the receptor for TGF $\alpha$ . In

addition, epithin expression appeared to be elevated in DCIS and in invasive breast carcinoma. A role for epithin in invasion and tumorigenesis is highly likely as epithin antibodies block TGF $\alpha$  release and invasion of BT-20 human breast cancer cells. Under the same conditions, invasion was found to be significantly inhibited by  $\alpha_1$ -AT. Our results suggest that serine proteases and serine protease inhibitors acting in concert may regulate the effects of E2 on breast cancer cell tumorigenesis.

## REFERENCES

- Arribas, J., Coodly, L., Vollmer, P. Kishimoto, T. K., Rose-John, S. Massagué, J. 1996 Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. *J Biol Chem* 271: 11376-11382.
- Arribas, J., Massagué, J. 1995 Transforming growth factor- $\alpha$  and beta-amyloid precursor protein share a secretory mechanism. *J Cell Biol* 128:433-441
- Baselga, J., Mendelsohn, J., Kim, Y.-M. and Pandiella, A. 1996 Autocrine regulation of membrane transforming growth factor- $\alpha$  cleavage. *J Biol Chem* 271:3279-3284.
- Bork, P. and Beckman G. 1993 The CUB domain. A widespread module in developmentally regulated proteins. *J Mol Biol* 231: 539-545.
- Brown, M.S., Herz, J., and Goldstein, J.L. 1997 *Nature* 388:629-630
- Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter W.J. 1979 *Biochemistry* 18: 5294-5299.
- Chung, D.W., Fujikawa, K., McMullen, B.A. and Davie, E.W. (1986) Human plasma prekallikrein, a zymogen to a serine protease that contains four tandem repeats. *Biochemistry* 25: 2410-2417.
- Claros, M.G. and von Heijne G 1994 TopPred II: an improved software for membrane protein structure predictions. *Comput. Appl Biosci* 10:685-686.
- De Jong, J.S., Van Diest, P.J., Van der Valk, P. and Baak, J.P. 1998 Expression of growth factors, growth inhibiting factors and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. *J Pathol* 184:53-57.
- Elvin, C.M., Whan, V. and Riddles, P.W. 1993 A Family of Serine Protease Genes Expressed in Adult Buffalo Fly. (*Haematobia irritans exigua*) *Mol. Gen. Genet.* 240:132-139.
- Finlay, T.H., Tamir, S., Kadner, S.S., Yavelow, J. and Levitz, M. 1993a  $\alpha_1$ -Antitrypsin and Anchorage-Independent Growth of MCF-7 Breast Cancer Cells. *Endocrinology* 133:996-1002.
- Frangioni, J.V. and Neel, B.G. (1993) Solubilization and Purification of Enzymatically Active Glutathione S-Transferase (pGEX) Fusion Proteins. *Analyt Biochem* 210:179-187.
- Fujikawa, K., Chung, D.W., Hendrickson, L.E., Davie, E.W. (1986) Amino acid sequence of human factor XI, a blood coagulation factor with four tandem repeats that are highly homologous with plasma prekallikrein. *Biochemistry*. 25:2417-2424.
- Harris, J.R., Lippman, M.E., Veronesi, U.V., Willett, W. 1992 *Breast Cancer N. Engl J Med* 327:319-328, 390-398, 473-480.
- Hofmann, K. & Stoffel 1993 Tmbase - A database of membrane spanning proteins. *Biol. Chem. Hoppe-Seyler* 347:166-168.
- Hooper, N.M, Karran, E.H. and Turner A.J. 1997 Membrane protein secretases. *Biochem J* 321:265-279.
- Kiessling, L.L. and Gordan, E.J 1998 Transforming the cell surface through proteolysis. *Chemistry & Biology* 5:R49-R62.
- Kim, M.G., Chen, C., Lyu, M.S., Cho, E.G., Park, D., Kozak, C., Schwartz, R.H. 1999 Cloning and Chromosomal Mapping of a Gene Isolated from Thymic Stromal Cells Encoding a New Mouse Type II Membrane Serine Protease, Epithin, Containing Four LDL Receptor Modules and Two cub Domains. *Immunogenetics* 49: 420-428.
- Kitamoto, Y., Veile, R.A., Donis-Keller, H. and Sadler, J.E. 1995 cDNA sequence and chromosomal localization of human enterokinase the proteolytic activator of trypsinogen. *Biochemistry* 34: 4562-4568.
- Kozak, M 1991 An analysis of vertebrate mRNA sequences: intimations of translational control *J Cell Biol* 115:887-903

- Lee, D.C., Fenton, S.E., Berkowitz, E.A. and Hissong, M.A. 1995 Transforming Growth Factor  $\alpha$ : Expression, Regulation, and Biological Activities. *Pharm. Rev.* 47:51-85.
- Lee, S.L., Dickson, R.B., Lin CY 2000 Activation of hepatocytes growth factor and urokinase and plasminogen activator by matriptase, an epithelial serine protease *J Biol Chem* 275:
- Leytus, S.P., Loeb, K.R., Hagen, F.S., Kurachi, K., Davie, E.W. (1988) A novel trypsin-like serine protease (hepsin) with a putative transmembrane domain expressed by human liver and hepatoma cells. *Biochemistry.* 27:1067-1074.
- Lin, C-Y., Anders, J., Johnson, M. Sang, Q.A. and Dickson, R.B. 1999 Molecular Cloning of cDNA for Matriptase, a Matrix-Degrading Protease with Trypsin-like Activity. *J. Biol. Chem.* 274:18231-18236.
- Massagué, J. and Pandiella, A. 1993 Membrane-Anchored Growth Factors. *Ann Rev Biochem* 62:515-541.
- Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y. and Kitamura, N. 1993 Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. *J. Biol. Chem.* 268:10024-10028.
- Paolino-Giacobino, A., Chen, H., Peitsch, M.C., Rossier, C. and Antronakis, S.C. 1997 Cloning of the TMPRSS2 gene, which encodes a novel serine protease with transmembrane, LDLRA, and SRCR domains and maps to 21q22.3 *Genomics* 44:309-320.
- Peschon, J.L., Slack, J.L., Reddy, P. et al 1998 An Essential Role for Ectodomain Shedding in Mammalian Development. *Science* 282: 1281-1284.
- Primakoff, P., Myles, D.G. 2000 The ADAM Gene Family: Surface Proteins with Adhesion and Protease Activity. *TIG* 16:83-87.
- Rawlings ND, Barrett AJ 1994 Families of serine peptidases. *Meth Enzymol* 244:19-61
- Sakanari, J.A., Staunton, C.E., Eakin, A.E, Craik, C.S. and McKerrow, J.H. (1989) Serine Proteases from Nematode and Protozoan Parasites: Isolation of Sequence Homologs Using Generic Molecular Probes. *Proc. Natl. Acad. Sci. USA.* 86:4863-4867.
- Sambrook, J., Fritsch, E.F., Maniatis, T. *Molecular Cloning: A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, New York, 1989.
- Takeuchi, T., Shuman, M.C., and Craik, C. 1999 Reverse biochemistry: Use of macromolecular protease inhibitors to dissect complex biological processes and identify a membrane-serine protease in epithelial cancer and normal tissue. *Proc. Natl. Acad. Sci. USA* 96:11054-11061.
- Takeuchi T, Harris JL, Huang W, Yan KW, Coughlin SR, Craik CS 2000 Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates. *J Biol Chem* 275: 26333-26342.
- Tamir, S., Kadner, S.S., Katz, J. and Finlay, T.H. 1990 Regulation of Antitrypsin and Antichymotrypsin Synthesis by MCF-7 Breast Cancer Cell Lines. *Endocrinology* 127:1319-1328.
- Yavelow J, Tuccillo A, Kadner SS, Katz J, Finlay TH. 1997  $\alpha_1$ -Antitrypsin Blocks Release of TGF $\alpha$  from MCF-7 Human Breast Cancer Cells. *J. Clin. Endo. Metabol* 82:745-752.
- Yu, J.X., Chao, L. and Chao, J. 1995 Molecular Cloning, tissue-specific expression and cellular localization of human prostasin mRNA. *J. Biol. Chem* 270:13483-13489.